Slov Vet Res 2022; 59 (1): 59–69
DOI 10.26873/SVR-1348-2022
UDC 639.2.09:579.841:579.25:615.015.8(620.1)

Original Research Article

ANTIMICROBIAL RESISTANCE AND VIRULENCE-ASSOCIATED GENES OF AEROMONADS ISOLATED FROM LAKE MANZALA WATER AND WILD NILE TILAPIA: IMPLICATIONS TO PUBLIC HEALTH AND THE LAKE MICROBIAL COMMUNITY

Hamza Mohamed Eid[1], Heba Sayed El-Mahallawy[2]*, Hassnaa Mahmoud Elsheshtawy[3], Amany Mahmoud Shalaby[4], Mera Mohamed Shetewy[5], Nada Hussein Eidaroos[1]

[1]Department of Bacteriology, Immunology, and Mycology, [2]Department of Animal Hygiene, Zoonoses, and Animal Behaviour and Management, [3]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt, [4]Department of Food Hygiene, Animal Health Research Institute, Port Said Branch, Port Said, [5]Free Veterinarian, Port Said, Egypt

*Corresponding author, Email: dr_ba1012_2@hotmail.com

Abstract: This study was conducted to investigate the prevalence, antimicrobial susceptibility, and molecular characterization of *Aeromonas* spp. from wild Nile tilapia from Lake Manzala and the lake water. Swabs from the surface, gills, and internal organs of apparently healthy Nile tilapia (n =100) and lake water (n = 25) were collected and examined bacteriologically for the presence of *Aeromonas* spp. The isolates obtained were tested for their susceptibility to 11 antimicrobial agents using the disk diffusion method. The presence of antibiotic resistance genes (bla_{TEM}, *sul1, tetA(A)*, and *aadA1*) and virulence genes (enterotoxins) (*alt, ast,* and *act*) was determined using conventional polymerase chain reaction. Overall, *Aeromonas* spp. were recovered from 69% of Nile tilapia (*Oreochromis niloticus*) samples and 80% of water samples. Four types of aeromonads were detected in all the samples examined, namely, *A. hydrophila*, *A. sobria, A. caviae,* and *A. schubertii*, with *A. hydrophila* predominating in both the fish and the lake water samples. The antimicrobial resistance profiles of the isolates showed very high resistance to ampicillin, penicillin, sulfamethoxazole/trimethoprim, and oxytetracycline and considerable resistance to streptomycin. However, all isolates were sensitive to cefotaxime. Molecular characterization revealed the presence of the *act* (100%) and *alt* (37.5%) genes, but *ast* was not found in any of the isolates. Specific amplification bands of the antimicrobial resistance genes bla_{TEM}, *sul1,* and *tetA(A)* were detected in all the tested isolates, whereas *aadA1* (12.5%) was found only in one isolate of *A. hydrophila* from Nile tilapia. The presence of these enterotoxigenic and resistant *Aeromonas* spp. in the fish and water samples from Lake Manzala could pose a potential public health threat to human consumers and fish handlers in the study area; moreover, these species carry a risk for the transfer of resistance genes to other microbial communities in the lake.

Key words: *Aeromonas hydrophila* complex; enterotoxin genes; antibiotic resistance genes; *Oreochromis niloticus*; Lake Manzala

Introduction

Lake Manzala is the largest and most important of Egypt's northern coastal lakes and is considered the most productive lake for fishing. The lake accounts for 32% of the total fish production from the lakes of Egypt (1), with the production of tilapia species alone accounting for >65% of the total Egyptian fish production (2). Over the last six decades, Lake Manzala has been subjected to various threats and has been contaminated with residues from domestic, industrial, mining, and agricultural effluents. The lake has evolved from a predominantly aquatic or estuarine ecosystem to an almost eutrophic freshwater system, which may have serious health, environmental,

Received: 2 June 2021
Accepted for publication: 7 January 2022

economic, and social consequences (3). Hence, poor water quality and the consequent decline in fish production in the lake have been reported (4).

Oreochromis niloticus is the most widely distributed freshwater fish species and one of the best known, cheapest, and most commonly available fish in Egypt. Owing to its high disease resistance, it can survive in harsh environmental conditions. Moreover, this fish species is preferred by aquaculture entrepreneurs because it can withstand a wide range of environmental factors, has active reproductive strategies, can feed at different trophic levels, and develops rapidly (5). The genus *Aeromonas* includes >30 different species, of which *A. hydrophila, A. caviae*, and *A. sobria* are of particular clinical importance (6). In humans, these species can cause several diseases, including septicemia, meningitis, wound infections, gastroenteritis, and pneumonia (7, 8). The virulence of *Aeromonas* spp. is multifactorial. Several factors, including endotoxins, enterotoxins, adhesins, hemolysins, cytotoxins, proteases, and lipases, have been detected in systemic and intestinal infections caused by aeromonads (9, 10). Remarkably, *Aeromonas* spp. can take up and transfer many genes located in genetic components such as plasmids, transposons, IS elements, genomic pathogen islands, and integron-associated gene cassettes. These genes may encode toxic elements, virulence factors, and antimicrobial resistance (11). These components are critical for the rapid transfer of genetic material between microbial communities. Therefore, environmental contamination is thought to provide an ideal medium for the selection of resistant species and the exchange of resistant genes by mobile genetic elements (12). Enterotoxins (also called exotoxins) secreted by aeromonads are one of the most important virulence factors (13); cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), and cytotonic heat-stable enterotoxin (*ast*) are a few examples (14, 15). The presence of virulence genes (enterotoxins) in *Aeromonas* spp. determines their degree of virulence (16). Owing to the frequent consumption of fish by people in our study area, much attention has been paid to the occurrence of antibiotic-resistant pathogens in fish and the associated risks to consumer health. Based on the existing data on this bacterial species, this study attempted to further investigate the antibiotic resistance profiles and virulence genes in extensively resistant aeromonads from apparently healthy Nile tilapia and water from Lake Manzala. We aimed to provide an up-to-date overview of the currently circulating isolates and to widen our knowledge of the likely public health implications.

Material and methods

Ethical statement

The collection and processing of samples for this study were reviewed and approved by the Scientific Research Committee and Bioethics Board of Suez Canal University, Faculty of Veterinary Medicine, Ismailia, Egypt.

Sampling

In total, 100 apparently healthy Nile tilapia were randomly netted at different locations in Lake Manzala. Swab samples (surface, gills, and internal organs) were collected from each fish. In addition, water samples (n = 25) were obtained in sterile, labeled, plastic screw-capped vials from the same areas where the fish were collected. All samples were immediately transported to the laboratory and processed under fully aseptic conditions for microbiological examination.

Isolation and identification of Aeromonas spp.

The isolation and identification of *Aeromonas* spp. from fish and water samples were performed in accordance with the standard procedures described previously (13, 17). Briefly, the samples were enriched in alkaline peptone water at 28°C for 18 h before inoculation onto Aeromonas agar medium (Oxoid, Hampshire, UK). Typical colonies suspected to be *Aeromonas* spp. were selected and purified for further identification. The isolates were identified to belong to the genus *Aeromonas* on the basis of Gram staining, motility tests, oxidase, glucose fermentation, catalase, and resistance to 2,4-diamino-6,7-diisopropylpteridine (O/129) (150 µg). A series of biochemical tests was used to identify the aeromonads at the species level. Pure colonies of the identified isolates were preserved on nutrient agar plates for further study.

Antimicrobial susceptibility and multiple antibiotic resistance (MAR) index value

Aeromonas spp. isolates were tested for susceptibility to the following 11 antimicrobial agents on Mueller–Hinton agar (Oxoid, Hampshire, UK) using the disk diffusion method (18) at the indicated concentrations: Ampicillin (AM, 10 µg); penicillin (P, 10 µg); streptomycin (S, 10 µg), sulfamethoxazole/ trimethoprim, (SXT, 25 µg); oxytetracycline (T, 30 µg); norfloxacin (NOR, 10 µg); cefotaxime (CTX, 30 µg); amikacin (AK, 30 µg); nalidixic acid (NA, 30 µg); gentamycin (CN, 10 µg); and chloramphenicol (C, 30 µg) (Bioanalysis®, Turkey). The tested strains were classified as sensitive or resistant based on the diameters of the inhibition zones around the disk. The MAR index was calculated using the following equation: MAR index = a/b; where, (a) is the number of antibiotics to which the isolate is resistant and (b) is the number of antibiotics to which the isolate has been exposed) (19).

Molecular characterization of Aeromonas spp. Isolates

Pure isolates were enriched by cultivating them in alkaline peptone water at 37°C for 24 h, and the QIAamp DNA mini kit was used to extract the genomic DNA in accordance with the manufacturer's instructions. For confirmation of the *Aeromonas* isolates, conventional polymerase chain reaction (PCR) was performed using *16S rRNA* primers, followed by the detection of enterotoxin genes (*alt*, *act*, and *ast*) and antibiotic resistance genes (bla_{TEM}, *sul1*, *tetA(A)*, and *aadA1*) corresponding to the agents to which the isolates were phenotypically resistant (Table 1). In all protocols, the reaction was performed in a total volume of 25 µL, which contained 12.5 µL dreamTaq Master Mix (Green PCR Master Mix (2×), (Thermo Scientific), 1 µL of each primer, 2 µL of DNA template, and 9.5 µL of DNase/RNAse-free water. The PCR cycling conditions were initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing for 40 s at the temperature indicated for each gene (50°C for 16S rRNA; 55°C for *alt*, *act*, and *ast*; 54°C for bla_{TEM} and *aadA1*; 50°C for *tetA(A)*; 60°C for *sul1*), and extension at 72°C for 30 s; and a final extension step at 72°C for 10 min. The amplified products were photographed after electrophoresis using an agarose gel (1.5%) stained with ethidium bromide (0.5 µg/m^1) against a 100-bp DNA ladder (Invitrogen, San Jose, CA).

Statistical analysis

The data were processed and analyzed using SPSS (IBM-SPSS Inc., Chicago, IL, USA). Examination of the data revealed that the Shapiro–Wilk P value was significant (*P* < 0.00*); hence, non-parametric data analysis was performed. The difference between the groups was considered significant when the *P* value of the chi-square test was <0.05.

Results

Prevalence of Aeromonas spp. in Nile tilapia and Lake Manzala water samples

Aeromonas bacterial spp. were found in 69% of *O. niloticus* (69/100) and 80% (20/25) of water samples. In total, 119 isolates belonging to the Aeromonas spp. were recovered from *O. niloticus*; *A. hydrophila* was the predominant species (54.62%), followed by *A. sobria* (26.05%), *A. caviae* (15.97%), and *A. schubertii* (3.36%). The same order was observed in the isolates from Lake Manzala too (n = 36). The predominant *Aeromonas* sp. was *A. hydrophila* (52.87%) (19/36), followed by *A. sobria* (27.78%) (10/36), *A. caviae* (16.76%) (6/36), and *A. schubertii* (2.78%) (1/36) (Table 2). The occurrence of *Aeromonas* spp. in different swabbing sites from Nile tilapia revealed that the surface showed the highest recovery rate (53.78%), followed by the gills (31.93%) and the internal organs (14.29%). The order of distribution of the different *Aeromonas* spp. followed the same pattern as in *O. niloticus* and lake water, with *A. hydrophila* being the predominant strain (Table 2). Statistically significant (*P* < 0.05) differences were found in the prevalence of *Aeromonas* spp. from Nile tilapia and lake water and from different swabbing sites.

Antimicrobial susceptibility and MAR index value of the identified Aeromonas isolates

As shown in Table 3, all of the 45 representative isolates of *Aeromonas* spp. tested from fish and lake water sources were multidrug resistant, i.e., resistant to ≥1 antibiotic falling under ≥3 antimicrobial classes. The isolates were highly resistant (100%) to ampicillin, penicillin, and sulfamethoxazole/

Table 1: Target genes, oligonucleotide primer sequences, and amplified product size specific for *Aeromonas* spp.

Target gene	Primer sequence (bp)	Amplified product size	Reference
16S rRNA	CTACTTTTGCCGGCGAGCGG TGATTCCCGAAGGCACTCCC	953 bp	(20)
act	GGGTGACCACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232 bp	(21)
ast	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	331 bp	
alt	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	442 bp	
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	(22)
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	(23)
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCAT	484 bp	(24)
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	

Table 2: Prevalence of *Aeromonas* spp. in the examined water samples and Nile tilapia, with special reference to their recovery rate from different swabbing sites of fish.

Aeromonas species	Water samples	Nile tilapia	Distribution in different swabbing sites of fish		
			Surface	Gills	Internal organs
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
A. hydrophila	19 (52.78)	65 (54.62)	34 (53.13)	21 (55.26)	10 (58.8)
A. sobria	10 (27.78)	31 (26.05)	17 (26.56)	10 (26.32)	4 (23.53)
A. caviae	6 (16.67)	19 (15.97)	11 (17.19)	6 (15.79)	2 (11.76)
A. schubertii	1 (2.78)	4 (3.36)	2 (3.13)	1 (2.6)	1 (5.88)
Total	36	119	64	38	17

Percent calculated in relation to the total of each column.

trimethoprim, followed by oxytetracycline (86.7%) and streptomycin (66.7%). However, all isolates were sensitive to cefotaxime (100%), with varying sensitivities to gentamycin (84.4%), chloramphenicol (80%), amikacin (75.6%), norofloxacin, and nalidixic acid (73.3%) (Table 3). These differences in the susceptibility of the four *Aeromonas* spp. to the different antimicrobial agents were statistically significant ($P < 0.05$). The MAR index values of the *Aeromonas* spp. ranged from 0.27–0.45 (Table 4).

Molecular confirmation and detection of enterotoxin genes and antibiotic resistance genes from isolates of the Aeromonas spp.

All tested *Aeromonas* isolates from the fish and water sources showed specific amplification bands at 953 bp when universal *Aeromonas* bacterial *16SrRNA* primers were used (Fig. 1 A). The detection of enterotoxin genes and antibiotic resistance genes for eight isolates, which showed an extensive drug resistance pattern, revealed the presence of the cytotonic heat-labile gene (*alt*) in three isolates (37.5%) and the cytotoxic enterotoxin gene (*act*) in all isolates. None of the isolates were positive for the cytotonic heat-stable enterotoxin (*ast*) gene (Table 5, Fig. 1 B, C, and D). With regard to the presence of the antibiotic resistance genes, bla_{TEM}, *sul1*, and *tetA(A)* were detected in all isolates of the *Aeromonas* spp. examined; however, the streptomycin-resistant gene (*aadA1*) was detected in only one *A. hydrophila* (12.5%) isolate from *O. niloticus* (Table 5, Fig. 2 A, B, C, and D).

Table 3: Antimicrobial resistance of *Aeromonas* spp. isolated from *O. niloticus* and Lake Manzala water samples

Antimicrobial agent (Conc.)	*A. hydrophila* n=18	*A. sobria* n=13	*A. caviae* n=9	*A. schubertii* n=5	Total n=45
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Ampicillin (AM) (10 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Penicillin (P) (10 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Streptomycin (S) (10 µg)	12 (66.7)	9 (69.2)	6 (66.7)	3 (60)	30 (66.7)
Sulphamethoxazole - trimethoprim (SXT) (25 µg)	18 (100.0)	13 (100.0)	9 (100.0)	5 (100)	45 (100.0)
Oxytetracyclin (T) (30 µg)	15 (83.3)	11 (84.6)	8 (88.9)	5 (100)	39 (86.7)
Cefotaxime (CTX) (30 µg)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Norofloxacin (NOR) (10 µg)	4 (22.2)	3 (23.1)	3 (33.3)	2 (40)	12 (26.7)
Amikacin (AK) (30 µg)	4 (22.2)	5 (38.5)	2 (22.2)	0 (0.0)	11 (24.4)
Gentamycin (CN) (10 µg)	5 (27.8)	1 (7.7)	1 (11.1)	0 (0.0)	7 (15.6)
Nalidixic acid (NA) (30 µg)	6 (33.3)	4 (30.8)	1 (11.1)	1 (20)	12 (26.7)
Chloramphenicol (C) (30 µg)	4 (22.2)	2 (15.4)	2 (22.2)	1 (20)	9 (20.0)

Table 4: MAR index value of *Aeromonas* species isolatedfrom Nile tilapia and Lake Manzala water samples

Strains	No. of isolates	Resistant antibiotics	MAR index	%
A. hydrophila	12	AM, P, SXT, S,T	0.45	66.6
	3	AM, P, SXT, T	0.36	16.6
	3	AM, P, SXT	0.27	16.6
A. sobria	9	AM, P, SXT, S,T	0.45	69.23
	2	AM, P, SXT, T	0.36	15.38
	2	AM, P, SXT	0.27	15.38
A. caviae	6	AM, P, SXT, S,T	0.45	66.6
	2	AM, P, SXT, T	0.36	16.6
	1	AM, P, SXT	0.27	16.6
A. schubertii	3	AM, P, SXT, S,T	0.45	60
	2	AM, P, SXT, T	0.36	30

AM: ampicillin, P: penicillin, S: streptomycin, SXT: sulphamethoxazole - trimethoprim, and T: oxytetracyclin

Table 5: Occurrence of *16SrRNA*, virulence (enterotoxin) genes and antimicrobial resistance genes in the examined *Aeromonas* species isolated from *O. niloticus* and Lake Manzala water samples

Aeromonas species	Enterotoxin genes			Antibiotic resistance genes			
	alt	*ast*	*act*	bla_{TEM}	*sul1*	*tetA(A)*	*aadA1*
A. hydrophila	-	-	+	+	+	+	+
A. hydrophila	+	-	+	+	+	+	-
A. hydrophila	+	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. sobria	-	-	+	+	+	+	-
A. caviae	-	-	+	+	+	+	-
A. schubertii	+	-	+	+	+	+	-
Total (%)	3(37.5%)	0 (0%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)	1 (12.5%)

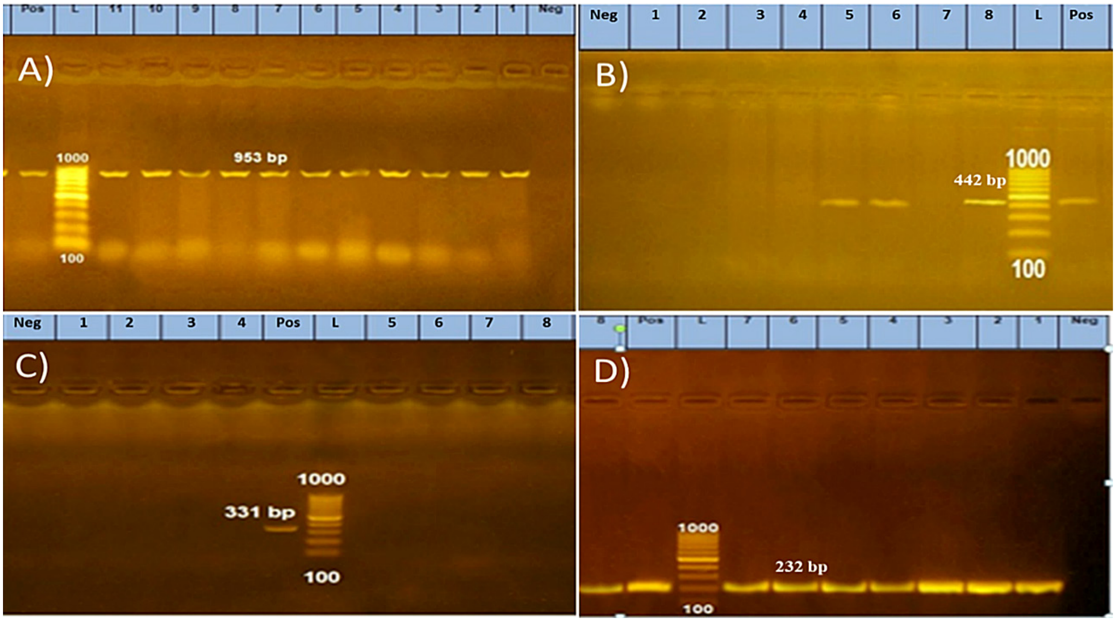

Figure 1: Agarose gel electrophoresis showing specific amplification product size of **A**) *16SrRNA* of *Aeromonas* species isolated from fish and water samples. Lane 1-8 are positive to genus *Aeromonas*, L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **B**) Cytotonic heat- labile enterotoxin (*alt*) gene. Lane 5, 6,and 8: positive for *(alt)* gene with a specific band at 442 bp. (*A. Schubertii* from *O. niloticus*, *A. hydrophila* from *O. niloticus* and *A. hydrophila* from lake water), L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **C**) Cytotoxic heat-stable enterotoxin *(ast)* gene. Lane 1-8: Negative for *(ast)* gene L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control. **D**) Cytotonic heat-stable enterotoxin *(act)* gene. Lane 1-8: positive with a specific *act* band at 232 bp. for *(act)* gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control, Neg: Negative control

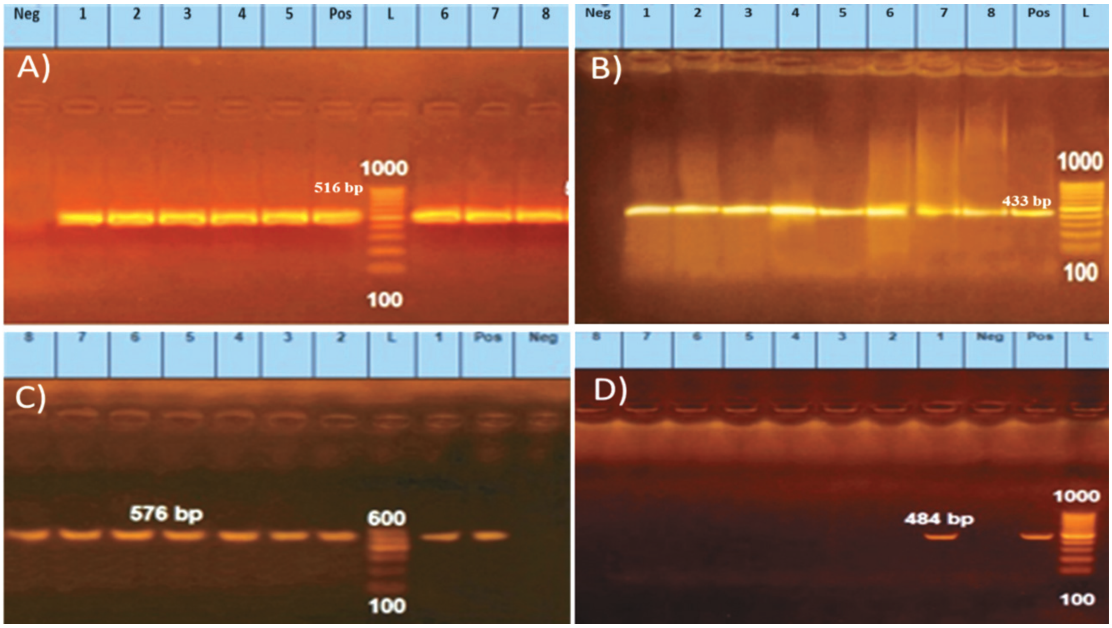

Figure 2: Agarose gel electrophoresis showing specific amplification product size of **A**) β- lactamase ampicillin resistance gene (*bla*$_{TEM}$ gene). Lane 1-8: Positive for (*bla*$_{TEM}$) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **B)** Sulfonamide resistance gene (*sul1* gene). Lane 1-8: Positive for (*sul1*) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **C)** Tetracycline resistance gene (*tet*A(A*)*gene). Lane 1-8: Positive for (*tet*A(A) resistance gene. L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control. **D)** Streptomycin resistance gene (*aadA1*gene). Lane 1: positive for (*aadA1*) resistance gene of *A. hydrophila* from *O. niloticus*, Lane 2-8: negative for (*aadA1*) resistance gene, L: 100 -1000 bp Molecular ladder, Pos: Positive control. Neg: Negative control.

Discussion

O. niloticus is one of the most common and valuable freshwater fish species in Egyptian commercial fisheries (25). Despite their wide distribution in the aquatic environment, aeromonads can cause disease in humans and fish depending on several environmental and host factors as well as the virulence of the organisms involved (26).

Similarly, *Aeromonas* spp. (84%) were isolated from Lake Manzala water (28). This high frequency of aeromonads may be attributed to the contamination of Lake Manzala water by large amounts of domestic sewage, agricultural, and industrial effluents (29). Motile aeromonads (*A. sobria*, *A. vernoii*, *A. jandae*, *A. cavae*, and *A. hydrophila*) have been described as the most common pathogens of carp and Nile tilapia in Egypt (30). The results of the present study showed that among the 119 aeromonad isolates obtained from *O. niloticus* and water, *A. hydrophila* was the most abundant species, followed by *A. sobria*, *A. caviae, and A. schubertii* (Table 2). This observation is in line with the results of a previous study on aeromonads from Lake Manzala (31). Similar results have been reported for Nile tilapia in Uganda where *A. hydrophila* (43.8%) and *A. sobria* (20.8%) were isolated (32). In addition, a high prevalence of *A. hydrophila* (47%) was found in 170 fish (100 from freshwater, 30 from the marine environment, and 40 from brackish water) from different farms in Alexandria, El-Behera, and Kafr Elsheikh in Egypt (33). Notably, *A. hydrophila* and *A. sobria*, which are most commonly associated with human infections (34, 35), were the most frequently isolated species from fish and water in this study.

In the current report, the highest recovery rate of aeromonads was recorded from the surface of the fish (53.78%, 64/119), followed by the gills (31.93%, 38/119) and internal organs (14.92%, 17/119), with *A. hydrophila* being the most common species (Table 2). Similar results have been reported previously when the recovery of *Aeromonas* spp. was higher in the skin of fish samples than in the internal organs and the gills (36). However, in a recent study in Egypt, the recovery rate of aeromonads was lower in the gills than in the internal organs (37). This finding may be related to the health status of the fish studied because the fish from the more recent study were clinically ill. In general, the isolation of different aeromonad species from fish and water provides information on the pollution rate as these species are widely distributed in surface waters and sewage systems (36).

The contamination of fish with resistant bacteria poses a serious threat to public health because genes encoding resistance can spread to other bacteria that are clinically relevant to humans. Consequently, antibiotic options for the treatment of common human infectious diseases have become increasingly limited, expensive, and ineffective owing to the emergence of antibiotic-resistant bacteria (29). A previous study has concluded that the genus *Aeromonas* can be used as an indicator bacterium to detect antibiotic resistance in the aquatic environment (38). In the present study, the examination of the susceptibility of 45 identified *Aeromonas* spp. isolates to 11 different antibiotics revealed that they were highly resistant to five antibiotics, namely, ampicillin, penicillin, sulfamethoxazole/trimethoprim (100% each), and oxytetracycline (86.7%) and considerably resistant to streptomycin (66.7%) (Table 3). However, in a recent report from Dakahlia, Egypt, *Aeromonas* spp. from farmed fish in private farms had high resistance to chloramphenicol (67.4%), amikacin (51.9%), and gentamicin (47.1%) but were sensitive to amoxicillin/clavulanic acid (73.3%) and trimethoprim/sulfamethoxazole (64.2%) (37). Since Lake Manzala is located in the northeastern part of the Nile Delta, between the Mediterranean Sea and the Suez Canal, it receives untreated and/or pretreated wastewater from the Bahr El-Baqar drain (100 km long), which is considered one of the most polluted drains in Egypt. This canal receives wastewater from two tributary canals (Bilbeis Canal and Qalubeya Canal) (39).

MAR is chiefly attributed to the improper use of antimicrobials. When the MAR index is >0.2, there is a high risk of bacterial contamination when antibiotics are used extensively (27). In the present study, the MAR index values for the four *Aeromonas* spp. described (Table 4) ranged from 0.27 to 0.45, which indicates the indiscriminate use of antibiotics. Such indiscriminate use may lead to the transfer of antibiotic resistance genes to other bacterial species and affect humans and fish, especially in areas with a high population density.

In this study, the eight isolates from Nile tilapia and lake water samples that showed extensive resistance patterns were screened for the presence of antibiotic resistance genes corresponding to the observed phenotypic resistance. Three genes (bla_{TEM}, *sul1*, and *tetA(A)*) were amplified from all eight *Aeromonas* spp. (Table 5). Similarly, bla_{TEM} antibiotic resistance genes were amplified from all *Aeromonas* isolates (40). However, in another study on Nile tilapia from Lake Temsah, Egypt, the bla_{TEM} resistance gene was detected to a lesser extent in *A. hydrophila* (73.68%, 14/19) and *A. sobria* (5.3%, 1/19) and to a much lesser extent in *A. hydrophila* (25%, 1/4) isolated from Nile tilapia from brackish water aquaculture (41). This difference in the prevalence of bla_{TEM} resistance genes may be due to the variations in the environments from which the *Aeromonas* spp. were isolated. The presence of β-lactamase genes in *Aeromonas* spp. from environmental sources is a complicated issue because it limits the therapeutic options for *Aeromonas* infections (42). In addition, the expression of the bla_{TEM} gene results in concurrent resistance to penicillins and broad-spectrum cephalosporins (43).

In the present study, the sulfonamide resistance gene (*sul1*) was detected in all *Aeromonas* isolates examined. However, the same gene was present at lower levels of 29.2% (44) and 11.5% (45) in other studies. Moreover, the presence of the *Sul1* gene has been reported to be different in *A. hydrophila* (80%, 8/10) and *A. caviae* (66.67%, 4/6) (46). These differences may be due to the presence of different Sul genes, such as *Sul1*, *Sul2*, and *Sul3*.

A specific amplification band of the *tetA(A)* gene was detected in all *Aeromonas* isolates tested, which agrees with the findings from another study (46). The streptomycin resistance gene, *aadA1*, was detected in only one strain of *A. hydrophila* (6.2%) from *O. niloticus*, which is less than that reported in other studies (30, 47). However, *aadA1* was detected in one strain of *A. hydrophila* from crayfish in Iran (48) and in seven of ten *A. hydrophila* isolates and in three of six *A. caviae* isolates in Egypt (30). These variations in the occurrence of the *aadA1* resistance gene could be due to the presence of other streptomycin resistance genes that were not investigated in this study, such as *aadA2* and *aadA5*.

Enterotoxin genes are very important for the pathogenicity of aeromonads. In the present study, the *act* gene was successfully amplified from the eight extensively drug resistant isolates from fish and water samples, but the *alt* gene was confirmed in only three isolates (Table 5). The prevalence of the *act* gene in this study was 100%, which agrees with another study (49). In contrast, other studies have shown different prevalence rates of 76.92% (50) and 65% (26) for the *act* gene in *Aeromonas* spp. The *act* gene was detected in 55% of *A. hydrophila* isolates, but it was not detected in *A. caviae* in another study (51). In this report, none of the isolates carried the cytotonic (*ast*) gene. This finding is in agreement with a previous result (26). However, the *ast* gene has been detected in the isolates of *Aeromonas* spp. in other studies at lower rates of 4% (52), 6% (50), and 10.25% (26). A higher occurrence of *alt* has previously been found in isolates of *A. hydrophila* (75%) but not in *A. caviae* (50) and in *A. sobria* but not in *A. caviae* (51). In general, biological tests are important for the evaluation of the virulence of spp. and to determine the pathogenicity of *Aeromonas* spp., which may pose a public health risk (53).

Conclusion

The ability of aeromonads to adapt to their environment is determined by their diverse virulence profiles. The presence of certain Aeromonas isolates with virulence and antimicrobial resistance properties in the same environment with other potentially pathogenic species poses a potential risk associated with the transfer of these virulence genes and antimicrobial resistance genes to other pathogenic microorganisms in Lake Manzala. Hence, these genes may reach humans and other animals through the food chain. The presence of such enterotoxigenic virulent strains on the surface and organs of fish poses a potential public health risk to fish handlers and human consumers in the study area.

Acknowledgments

The authors declare that they have no conflict of interest.

This research was not financially supported by any public, commercial, or nonprofit entity.

References

1. El-Bokhty E-A, Amin A. Current status of Liza ramada (Risso, 1810)(Mugilidae) caught by

trammel net (Ballah) at El-Gamil region, Manzala Lake, Egypt. Egypt J Aquat Biol Fish 2020; 24(1): 281–308.

2. GAFRD. Fish statistics year book, 2016. Cairo : The General Authority for fish Resources Development, 2016.

3. Ismail A, Hettiarachchi H. Environmental damage caused by wastewater discharge into the Lake Manzala in Egypt. Am J Biosci Bioeng 2017; 5(6): 141–50.

4. Elnaggar AA, El-Alfy MA. Physiochemical properties of water and sediments in Manzala Lake, Egypt. J Environ Sci 2016; 45(2):157–74.

5. Abdel-Ghaffar F, El-Toukhy A, Al-Quraishy S, et al. Five new myxosporean species (Myxozoa: Myxosporea) infecting the Nile tilapia *Oreochromis niloticus* in Bahr Shebin, Nile Tributary, Nile Delta, Egypt. Parasitol Res 2008; 103(5): 1197–205.

6. Persson S, Al-Shuweli S, Yapici S, Jensen JN, Olsen KE. Identification of clinical *Aeromonas* species by rpoB and gyrB sequencing and development of a multiplex PCR method for detection of *Aeromonas hydrophila*, *A. caviae, A. veronii*, and *A. media*. J Clin Microbiol 2015; 53(2): 653–6.

7. Galindo CL, Sha J, Fadl AA, Pillai LL, Chopra AK. Host immune responses to Aeromonas virulence factors. Curr Immunol Rev 2006; 2(1): 13–26.

8. Galindo CL, Chopra AK. *Aeromonas* and *Plesiomonas* species. In: Doyle MP, Beunchal LR, eds. Food microbiology: fundamentals and frontiers 3rd ed. Washington : ASM Press, 2007: 381–400.

9. Guerra IM, Fadanelli R, Figueiró M, et al. *Aeromonas* associated diarrhoeal disease in south Brazil: prevalence, virulence factors and antimicrobial resistance. Braz J Microbiol 2007; 38(4): 638–43.

10. Tomás J. The main *Aeromonas* pathogenic factors. ISRN Microbiol 2012; 2012: e256261. doi: 10.5402/2012/256261

11. Piotrowska M, Przygodzińska D, Matyjewicz K, Popowska M. Occurrence and variety of β-lactamase genes among *Aeromonas* spp. isolated from urban wastewater treatment plant. Front Microbiol 2017; 8: e863. doi: 10.3389/fmicb.2017.00863

12. Zhong C, Han M, Yang P, et al. Comprehensive analysis reveals the evolution and pathogenicity of *Aeromonas*, viewed from both single isolated species and microbial communities. mSystems 2019; 4(5): e00252-19. doi: 10.1128/mSystems.00252-19.

13. Kingombe CIB, D'Aoust JY, Huys G, Hofmann L, Rao M, Kwan J. Multiplex PCR method for detection of three *Aeromonas* enterotoxin genes. Appl Environ Microbiol 2010; 76(2): 425–33.

14. Sha J, Kozlova E, Chopra A. Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun. 2002; 70(4): 1924–35.

15. Chopra A, Xu X-J, Ribardo D, et al. The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect Immun 2000; 68(5): 2808–18.

16. Albert MJ, Ansaruzzaman M, Talukder KA, et al. Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. J Clin Microbiol 2000; 38(10): 3785–90.

17. Janda JM, Abbott SL. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 2010; 23(1): 35–73.

18. CLSI. Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement. CLSI Document M100-S25, 2015.

19. Rasmussen-Ivey CR, Figueras MJ, McGarey D, Liles MR. Virulence factors of *Aeromonas hydrophila*: in the wake of reclassification. Front Microbiol 2016; 7: e1337. doi: 10.3389/fmicb.2016.01337

20. Gordon L, Giraud E, Ganière JP, et al. Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. J Appl Microbiol 2007; 102(4): 1167–76.

21. Nawaz M, Khan SA, Khan AA, et al. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol 2010; 27(3): 327–31.

22. Colom K, Pérez J, Alonso R, Fernández -Aranguiz A, Lariño E, Cisterna R. Simple and reliable multiplex PCR assay for detection of bla TEM, bla SHV and bla OXA–1 genes in Enterobacteriaceae. FEMS Microbiol Lett 2003; 223(2): 147–51.

23. Ibekwe AM, Murinda SE, Graves AK. Genetic diversity and antimicrobial resistance of *Escherichia coli* from human and animal sources uncovers multiple resistances from human sources. PLoS One 2011; 6(6): e20819. doi: 10.1371/journal.pone.0020819.

24. Randall L, Cooles S, Osborn M, Piddock L, Woodward MJ. Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of *Salmonella*

enterica isolated from humans and animals in the UK. J Antimicrob Chemother 2004; 53(2): 208–16.

25. El Deen AN, Dorgham-Sohad M, Hassan-Azza H, Hakim A. Studies on *Aeromonas hydrophila* in cultured *Oreochromis niloticus* at Kafr El Sheikh Governorate, Egypt with reference to histopathological alterations in some vital organs. World J Fish Mar Sci 2014; 6(3): 233–40.

26. Rather M, Willayat M, Wani S, Hussain S, Shah S. Enterotoxin gene profile and molecular epidemiology of *Aeromonas* species from fish and diverse water sources. J Appl Microbiol 2019; 127(3): 921–31.

27. Salem M, Zharan E, Saad R, Zaki V. Prevalence, molecular characterization, virulotyping, and antibiotic resistance of motile aeromonads isolated from Nile tilapia farms at northern Egypt. Mansoura Vet Med J 2020; 21(1): 56–67.

28. El-Sayed EM, Shalaby AM, Saleh MH, Abo El-Kheir RE-S. Genotypic identification and evaluation of several selective media for recovery of *Aeromonas* spp. from different sources. Egypt Acad J Biol Sci G Microbiol 2016; 8(1): 13–31.

29. Zaky MM, Ibrahim ME. Screening of bacterial and fungal biota associated with *Oreochromis niloticus* in Lake Manzala and its impact on human health. Health 2017; 9(4): 697–714.

30. Abd El-Tawab A, Ashraf, A Saleh O, M Awad S, M Absi H. Phenotypic and genotypic characters on Aeromonas species causing mortality in fishes. Benha Vet Med J 2018 ;35(1): 285–300.

31. Eid H, Soliman Z, Hanafy AS. Molecular detection of some virulence genes in *Aeromonas* species isolated from fishes and water of Manzala Lake. Suez Canal Vet Med J SCVMJ. 2019; 24(2): 231–43.

32. Wamala SP, Mugimba KK, Mutoloki S, et al. Occurrence and antibiotic susceptibility of fish bacteria isolated from *Oreochromis niloticus* (Nile tilapia) and *Clarias gariepinus* (African catfish) in Uganda. Fish Aquat Sci 2018; 21: e6. doi:10.1186/s41240-017-0080-x

33. Dahdouh B, Basha O, Khalil S, Tanekhy M. Molecular characterization, antimicrobial susceptibility and salt tolerance of *Aeromonas hydrophila* from fresh, brackish and marine fishes. Alexandria J Vet Sci 2016; 48(2): 46–53. doi:10.5455/ajvs.208107

34. Ashbolt NJ. Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology 2004; 198(13): 229–38.

35. Dumontet S, Pasquale V, Mancino M, Normanno G, Krovacek K. Incidence and characterisation of *Aeromonas* spp. in environmental and human samples in southern Italy. New Microbiol 2003; 26(2): 215–25.

36. Maimona S, Sabiel Y. Detection of the causative agents of bacterial fish septicemia of tilapia and clarais in Khartoum state. Int J Recent Sci Res 2015; 6: 4374–7.

37. Saleh A, Elkenany R, Younis G. Virulent and multiple antimicrobial resistance *Aeromonas hydrophila* isolated from diseased Nile tilapia fish (*Oreochromis niloticus*) in Egypt with sequencing of some virulence-associated genes. Biocontrol Sci 2021; 26(3): 167–76.

38. Baron S, Granier SA, Larvor E, et al. *Aeromonas* diversity and antimicrobial susceptibility in freshwater: an attempt to set generic epidemiological cut-off values. Front Microbiol 2017; 8: e503. doi: 10.3389/fmicb.2017.00503

39. El-Fadaly H, El-Kadi S, El-Kholy S. Chemical and microbiological examinations of water and fish taken from Manzala Lake of *Damietta gavernorate*, Egypt. Am J Food Sci Health 2019; 5(2): 50–60.

40. Chung T, Yi S, Kim B, Kim W, Shin G. Identification and antibiotic resistance profiling of bacterial isolates from septicaemic soft-shelled turtles (*Pelodiscus sinensis).* Vet Med (Praha) 2017; 62(3): 169–77.

41. Shawky LAI. Studies on virulent and antibiotic resistant genes of *Aeromonas* species isolated from fish: thesis. Ismailia, Egypt : Faculty of Veterinary medicine, Suez canal University, 2015.

42. Roges EM, Gonçalves VD, Cardoso MD, et al. Virulence-associated genes and antimicrobial resistance of *Aeromonas hydrophila* isolates from animal, food, and human sources in Brazil. BioMed Res Int 2020; 2020: e1052607. doi: 10.1155/2020/1052607

43. Weldhagen GF, Poirel L, Nordmann P. Ambler class A extended-spectrum β-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. Antimicrob Agents Chemother 2003; 47(8): 2385–92.

44. Okolie CA. Characterization of antimicrobial resistance genes of *Aeromonas* spp. isolated from fish and investigation of phytochemical treatment efficacy against resistant isolates: Master dissertation. Durban, South Africa : Uni-

versity of KwaZulu-Natal; 2015.

45. Nwaiwu O, Aduba CC. An in silico analysis of acquired antimicrobial resistance genes in *Aeromonas* plasmids. AIMS Microbiol 2020; 6(1): 75–91.

46. Abd El Tawab AA, Maarouf AAA, El Hofy FI, Salimand AO, El Mougy EEA. Molecular studies on antibiotic resistant genes of Aeromonas species isolated from fish. Nat Sci 2017; 15(12): 90–7.

47. Ndi O, Barton M. Incidence of class 1 integron and other antibiotic resistance determinants in *Aeromonas* spp. from rainbow trout farms in Australia. J Fish Dis 2011; 34(8): 589–99.

48. Ranjbar R, Salighehzadeh R, Sharifiyazdi H. Antimicrobial resistance and incidence of integrons in *Aeromonas* species isolated from diseased freshwater animals and water samples in Iran. Antibiotics 2019; 8(4): e198. doi: 10.3390/antibiotics8040198

49. Zhang DX, Kang YH, Song MF, et al. Identity and virulence properties of *Aeromonas* isolates from healthy Northern snakehead (*Channa argus*) in China. Lett Appl Microbiol 2019; 69(2): 100–9.

50. Khor WC, Puah SM, Tan JAMA, Puthucheary S, Chua KH. Phenotypic and genetic diversity of *Aeromonas* species isolated from fresh water lakes in Malaysia. PLoS One 2015; 10(12): e0145933. doi: 10.1371/journal.pone.0145933

51. De Silva B, Hossain S, Wimalasena S, Pathirana H, Heo GJ. Putative virulence traits and antibiogram profile of *Aeromonas* spp. isolated from frozen white–leg shrimp (*Litopenaeus vannamei*) marketed in Korea. J Food Saf. 2018; 38(4): e12470. doi: I:10.1111/jfs.12470

52. Didugu H, Thirtham M, Nelapati K, et al. A study on the prevalence of *Aeromonas* spp. and its enterotoxin genes in samples of well water, tap water, and bottled water. Vet World 2015; 8(10): 1237–42.

53. Hu M, Wang N, Pan Z, Lu C, Liu Y. Identity and virulence properties of *Aeromonas* isolates from diseased fish, healthy controls and water environment in China. Lett Appl Microbiol 2012; 55(3): 224–33.

PROTIMIKROBNA ODPORNOST IN Z VIRULENCO POVEZANI GENI BAKTERIJE *AEROMONAS* IZOLIRANIH IZ JEZERA MANZALA IN DIVJE NILSKE TILAPIJE: POSLEDICE NA JAVNO ZDRAVJE IN NA JEZERSKO MIKROBNO ZDRUŽBO

H. M. Eid, H. S. El-Mahallawy, H. M. Elsheshtawy, A. M. Shalaby, M. M. Shetewy, N. H. Eidaroos

Izvleček: Namen te študije je bil raziskati razširjenost in protimikrobno občutljivost ter molekularno karakterizirati bakterijo *Aeromonas spp.*, izolirane iz divje nilske talapije in vode iz jezera Manzala. Zbrali smo brise površine, škrg in notranjih organov navidezno zdrave nilske tilapije (n = 100) in vzorce vode (n = 25), ki smo jih bakteriološko pregledali na prisotnost bakterije *Aeromonas spp.* Pridobljene izolate smo testirali na občutljivost za 11 protimikrobnih sredstev z metodo difuzije diska. Prisotnost genov za odpornost proti antibiotikom (*bla*$_{TEM}$, *sul1*, *tetA(A)* in *aadA1*) in genov za virulenco (enterotoksini; *alt*, *ast* in *act*) smo določili z običajno verižno reakcijo s polimerazo. Skupno smo *Aeromonas spp.* odkrili v 69% vzorcev nilske tilapije in 80% vzorcev vode. V vseh pregledanih vzorcih smo odkrili štiri vrste bakterij in sicer *A. hydrophila, A. sobria, A. caviae*, in *A. schubertii*, od katerih je *A. hydrophila* prevladovala v vzorcih rib in jezerske vode. Profili protimikrobne odpornosti izolatov so pokazali zelo visoko odpornost na ampicilin, penicilin, sulfametoksazol/trimetoprim in oksitetraciklin ter znatno odpornost na streptomicin. Izolati so bili občutljivi na cefotaksim. Molekularna karakterizacija je pokazala prisotnost genov *act* (100%) in *alt* (37.5%). Gena *ast* nismo našli v nobenem izolatu. Geni za protimikrobno odpornost *bla*$_{TEM}$, *sul1* in *tetA(A)* so bili prisotni pri vseh testiranih izolatih, medtem ko je bil gen *aadA1* (12.5%) najden samo pri enem izolatu *A. hydrophila* iz nilske tilapije. Prisotnost enterotoksičnih in odpornih bakterij *Aeromonas spp.* v vzorcih rib in vode iz jezera Manzala bi lahko predstavljala nevarnost za javno zdravje potrošnikov in oseb, ki rokujejo z ribami na območju študije; poleg tega te vrste predstavljajo tveganje za prenos genov za odpornost na druge mikrobne skupnosti v jezeru.

Ključne besede: *Aeromonas hydrophila* kompleks; geni za enterotoksin; geni za rezistenco na antibiotike; *Oreochromis niloticus*; jezero Manzala